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C20 elongase activity in the anadromous teleost meagre
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Title

Long-chain polyunsaturated fatty acid biosynthesis in teleosts: Functional characterisation of fatty acyl desaturase (Fads2) and Elovl5 elongase in the meagre *Argyrosomus regius* (Asso, 1801)

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Summary

The meagre, *Argyrosomus regius*, is a carnivorous fish with great potential to diversify finfish aquaculture in the Mediterranean. However, currently nothing is known about their essential fatty acid requirements. Meagre are marine fish but also display anadromous behaviour migrating to river estuaries to spawn, and thus may provide insight to the influence of diadromy on biosynthetic ability for long-chain polyunsaturated fatty acid (LC-PUFA). Our primary aim was to characterise key cDNAs (fatty acyl desaturases and elongases) of the biosynthetic pathway as a key step to establish the capacity of meagre for LC-PUFA biosynthesis from shorter chain PUFA. The cDNA sequences of a fatty acyl desaturase (Fads) and elongase of very long-chain fatty acids (Elovl) were obtained using PCR-based methodologies, and function of the proteins investigated by expression of the coding sequences of the putative desaturase and elongase in the yeast *Saccharomyces cerevisiae*. The tissue distribution of both cDNAs was studied by reverse transcription PCR. Our results demonstrated that meagre possess at least one fatty acyl desaturase and one elongase involved in the endogenous production of LC-PUFA. The meagre desaturase and elongase were identified as orthologues of Fads2 and Elovl5, respectively. Functionally, the desaturase had dual $\Delta 6/\Delta 8$ activity, whereas the elongase exhibited high elongation efficiency for C₁₈ and C₂₀ PUFA with low activity towards C₂₂ PUFA. However, we also showed that the meagre Elovl5 elongated 16:3n-3 to 18:3n-3, the first time that C₁₆ elongation activity had been demonstrated for a fish elongase. Similar to other marine teleosts, expression of *fads2* and *elovl5* transcripts was highest in brain. The functions and expression of the meagre Fads2 and Elovl5 proteins suggest that the meagre has a 'marine type' LC-PUFA biosynthetic pathway, and that its anadromous behaviour has no major influence.

Keywords: Elongase of very long-chain fatty acids; essential fatty acids; fatty acyl desaturase; meagre; omega-3; polyunsaturated fatty acids.

Introduction

Increased dietary intake of n-3 ('omega-3') long-chain polyunsaturated fatty acids (LC-PUFA) in humans has beneficial effects in several inflammatory and pathological conditions, including cardiovascular and neurological diseases (see Campoy et al., 2012; Delgado-Lista et al., 2012; Gil et al., 2012; Miles and Calder, 2012; Rangel-Huerta et al., 2012). Fish are the major dietary source for humans of n-3 LC-PUFA (Tur et al., 2012) and, with declining fisheries worldwide (Worms et al., 2006), farmed fish constitute an ever-increasing proportion of fish in the human food basket amounting to almost 50% in 2010 (FAO, 2011). Until now, high levels of n-3 LC-PUFA in farmed fish have been obtained by the use in the feeds of fish oils (FO), themselves derived from industrial marine fisheries, but this is not sustainable and will constrain continuing growth of aquaculture (Tacon and Metian, 2008). In consequence, alternatives to FO are required, with vegetable oils (VO) as prime candidates (Turchini et al., 2010). However, VO are rich in C₁₈ PUFA, but devoid of the n-3 LC-PUFA abundant in FO (Sargent et al., 2002). Feeding fish with VO-rich diets can have important consequences for the human consumer as it lowers the n-3 LC-PUFA content of farmed fish compromising their nutritional value (Turchini et al., 2010).

Biosynthesis of LC-PUFA in vertebrates involves sequential desaturation and elongation of precursor PUFA, linoleic acid (LOA; 18:2n-6) and α -linolenic acid (ALA; 18:3n-3). Synthesis of arachidonic acid (ARA; 20:4n-6) from LOA and eicosapentaenoic acid (EPA; 20:5n-3) from ALA utilises the same enzymes and pathways. The conventional pathway of LC-PUFA biosynthesis from the C₁₈ PUFA, LOA or ALA, involves a Δ 6 desaturation to 18:3n-6/18:4n-3 that are elongated to 20:3n-6/20:4n-3 followed by Δ 5 desaturation to ARA/EPA (Cook and McMaster, 2004). Recently, however, it was shown that an alternative pathway involving initial elongation of LOA or ALA followed by Δ 8 desaturation (an inherent ability of some Δ 6 desaturases) may be possible (Monroig et al., 2011a). Docosahexaenoic acid (DHA; 22:6n-3) synthesis from EPA can also follow different

pathways. The “Sprecher pathway”, involving two sequential elongation steps to 24:5n-3 and a second $\Delta 6$ desaturation (again by the same $\Delta 6$ desaturase) followed by peroxisomal chain shortening, was regarded as the vertebrate pathway (Sprecher, 2000). However, the recent discovery of vertebrate fatty acyl desaturases with $\Delta 4$ activity has indicated that a more direct route in which the elongation product of EPA, i.e. docosapentaenoic acid (DPA; 22:5n-3) can be desaturated to DHA, is also possible, at least in some teleost fish species (Li et al., 2010; Morais et al., 2012).

The meagre, *Argyrosomus regius*, a carnivorous teleost of the Sciaenidae family (drums and croakers) has been identified as a potential new species for Mediterranean aquaculture (Poli et al., 2001a,b) and, in recent years, production has risen reaching around 10,000 tonnes in 2010 (Monfort, 2010; FAO, 2011). In the wild, meager can reach lengths of 2 m and weights of 50 kg (Monfort, 2010) and, in farming, can rapidly attain relatively large commercial sizes, which is advantageous for processing and could provide a unique market niche compared to sea bream and bass (Poli et al., 2001a). In addition, meagre flesh is lean, even when grown intensively on high fat diets, and is known for having a high content of n-3 LC-PUFA (Poli et al., 2001a,b).

As with all vertebrates, PUFA are essential in the diet of fish, but requirements vary with species (Bell and Tocher, 2009; Tocher, 2003). Whereas C₁₈ PUFA satisfy the essential fatty acid (EFA) requirements of freshwater species, many marine fish have a dietary requirement for preformed LC-PUFA such as EPA and DHA (Tocher, 2010). Interestingly, Atlantic salmon, an anadromous species, living in the sea as an adult but returning to freshwater to spawn, displays a freshwater pattern (Tocher, 2003). Like Atlantic salmon, meagres also display anadromous behaviour, migrating from deep waters of the North-eastern Atlantic and Mediterranean sea to coastal areas in the spring to spawn in river estuaries (Monfort, 2010). Therefore, the LC-PUFA biosynthetic capability of meager is of considerable interest not only as it is a novel farmed fish species whose EFA requirements are unknown, but also as it may

provide further insight to the relationship between diadromy and LC-PUFA biosynthetic pathways or mechanisms.

The different EFA requirements between freshwater/salmonids and marine fish has been associated with differences in the complement of fatty acyl desaturase and elongase genes that participate in the biosynthetic pathways of essential LC-PUFA like ARA, EPA and DHA (Agaba et al., 2004, 2005; Hastings et al., 2001, 2005; Monroig et al., 2009, 2010a,b; Tocher et al., 2006; Zheng et al., 2004, 2005a). More specifically, it has been established that the dependence of many marine fish on dietary LC-PUFA is known to be caused by deficiency in one or more key enzymes required for their endogenous biosynthesis (Tocher, 2003, 2010). Our primary aim is to gain a better understanding of the EFA requirements of meagre that will allow us to optimise the efficient use of sustainable plant-based alternatives in the incipient meagre aquaculture industry while maintaining the nutritious n-3 LC-PUFA content for the human consumer. The specific objectives of the present study are the cDNA cloning, functional characterisation and tissue distributions of a fatty acyl desaturase and PUFA elongase involved in the LC-PUFA biosynthesis pathways of meagre.

Materials and methods

Tissue samples

One single (male) meagre (~150 g) that had been maintained at the facilities of the Instituto de Acuicultura Torre de la Sal (IATS-CSIC), Spain, was anaesthetised and sacrificed with an overdose of MS-222 (Sigma-Aldrich, Alcobendas, Spain). Tissues including liver, gill, stomach, pyloric caeca, spleen, testicle, kidney, heart, muscle, gallbladder, skin, brain and eye were collected immediately and frozen at -80 °C until further analysis.

Desaturase and elongase cDNA cloning

126 Total RNA was extracted from meagre tissues using TRI Reagent (Sigma-Aldrich)
 127 following manufacturer's instructions. cDNA was thereafter synthesised from 2 µg total RNA
 128 using a GoScriptTM Reverse Transcription System (Promega, Madison, WI, USA) primed
 129 with random primers. In order to amplify the first fragment of desaturase cDNA, several fatty
 130 acyl desaturase 2 (*fads2*) -like sequences from teleosts including *Oncorhynchus masou*
 131 (AB074149.1), *Thunnus thynnus* (HQ214238.1), *Siniperca chuatsi* (EU683737.1), *Solea*
 132 *senegalensis* (JN673546.1), *Sparus aurata* (GQ162822.1) and *Rachycentron canadum*
 133 (FJ440238.1) were aligned with BioEdit v7.0.9 (Tom Hall, Department of Microbiology,
 134 North Carolina State University, USA). Similarly, a first fragment of the meagre elongase was
 135 isolated with primers designed on conserved regions among elongase of very long-chain fatty
 136 acids 5 (Elovl5) sequences of *Danio rerio* (NM_200453.1), *Anguilla japonica* (EU719614.1),
 137 *Salmo salar* (NM_001136552.1), *Sparus aurata* (GQ162823.1), *Lates calcarifer*
 138 (GQ214180.1), *Siganus canaliculatus* (GU597350.1) and *Gadus morhua* (AY660881.2).
 139 Those alignments enabled the design of the primers UNIDF (5'-
 140 GGAGAGGAYGCCACGGAGG-3') and UNIDR (5'- GTCCRCTGAACCAGTCGTTGAA-
 141 3') for the desaturase, and UNIE5F (5'- CATGGATGGGYCCMAGAGATC-3') and
 142 UNIE5R (5'- GTCTGAATGTAGAAGTTTGAGAAAAG-3') for the elongase. The primer
 143 pairs UNIDF-UNIDR and UNIE5F-UNIE5R were used for polymerase chain reaction (PCR)
 144 with KAPA Taq Hotstart (Kapa Biosystems, Boston, MA, USA), and using a mixture of
 145 cDNA from liver, brain and eye as template. The PCR consisted of an initial denaturing step
 146 at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C
 147 for 30 s, extension at 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 1 min.
 148 The PCR fragments were confirmed as positive by sequencing (DNA Sequencing Service,
 149 IBMCP-UPV, Valencia, Spain). The desaturase and elongase open reading frame (ORF)
 150 fragments were further extended by 3' rapid amplification of cDNA ends (RACE) PCR
 151 (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington, UK) through a

two-round PCR approach using a combination of gene-specific forward primers and RACE kit reverse primers (see Table 1 for details of primers used for cDNA cloning). The first round PCR was performed combining the gene-specific forward primers ARDF1 (desaturase) and ARE5F1 (elongase) with the adapter-specific 3'RACE OUTER primer, with an initial denaturing step at 95 °C for 2 min, followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 2 min 30 s, followed by a final extension at 72 °C for 1 min (KAPA Taq). First round PCR products were used as template for nested PCR with primers ARDF2 (desaturase) and ARE5F2 (elongase) and 3'RACE INNER in a 32-cycle reaction under the same thermal conditions as above. The 5' ends of the meagre desaturase and elongase cDNAs were obtained by PCR using the meagre gene-specific reverse primers ARDR (desaturase) and ARE5R (elongase) with the forward primers NMDesF (desaturase) and LCE5F (elongase), the latter based on the sequences of the corresponding orthologues found in the closely related croaker species *Nibea mitsukurii* (for desaturase) and *Larimichthys crocea* (for elongase). PCR products were cloned into pGEM-T Easy Vector (Promega) and sequenced as above.

(TABLE 1)

Sequence and phylogenetic analyses

The amino acid (aa) sequence identities of the *A. regius* putative Fads2 and Elovl5 proteins were compared to those of orthologues from other fish and tetrapods (mammals, birds and amphibians) using the EMBOSS Needle Pairwise Sequence Alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Phylogenetic analyses of the aa sequences deduced from the *fads2*- and *elovl5*-like cDNA from meagre and those from other vertebrates were carried out by constructing two trees using the neighbor-joining method (Saitou and Nei 1987), with confidence in the resulting tree branch topology measured by bootstrapping

through 10,000 iterations. The $\Delta 6$ desaturase and the PUFA elongase sequences from *Mortierella alpina* were used as outgroup sequences to construct both rooted trees.

Functional characterisation of the meagre Fads2 and Elovl5 by heterologous expression in yeast

PCR fragments corresponding to the ORF of the meagre Fads2 and Elovl5 were amplified from a mixture of cDNA (liver, brain and eye) by PCR with the primer pairs ARDVF-ARDVR (desaturase) and ARE5VF-ARE5VR (elongase) containing *EcoRI* and *XhoI* restriction sites (underlined in Table 1). PCR was performed with the high fidelity *Pfu* DNA Polymerase (Promega) under conditions consisting of an initial denaturing step at 95 °C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 3 min (desaturase) or 2 min (elongase), followed by a final extension at 72 °C for 5 min. The PCR products were subsequently purified (Illustra GFX PCR DNA/Gel Band Purification Kit, GE Healthcare), digested with the corresponding restriction enzymes (Promega) and ligated into a similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, UK). The purified plasmids (GenElute™ Plasmid Miniprep Kit, Sigma) containing the meagre *fads2* and *elovl5* ORF were then used to transform *Saccharomyces cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and selection of yeast with recombinant pYES2-*fads2* or pYES2-*elovl5* plasmids, and yeast culture were performed as described in detail previously (Agaba et al., 2004; Monroig et al., 2012a). Briefly, yeast transformed with pYES2-*fads2* and pYES2-*elovl5* were grown in *S. cerevisiae* minimal medium-^{uracil} broth and diluted to OD600 of 0.4 in one single Erlenmeyer flask for each potential substrate assayed. In order to test the ability of the meagre *fads2* cDNA to desaturate PUFA substrates, the transgenic yeast were grown in medium supplemented with one of the following substrates: 18:4n-3, 18:3n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and 22:4n-6. Similarly, the meagre Elovl5 was functionally characterised

by growing yeast transformed with pYES2-*elovl5* in medium containing one of the following substrates: 16:3n-3, 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 and 22:4n-6. The fatty acid (FA) substrates were added to the yeast cultures at final concentrations of 0.5 (C18), 0.75 (C20) and 1.0 (C22) mM as uptake efficiency decreases with increasing chain length (Zheng et al., 2009). Yeast transformed with empty pYES2 were also grown in presence of PUFA substrates as control treatments. After 2-days culture at 30 °C, yeast were harvested, washed, and total lipid extracted by homogenisation in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxy toluene (BHT) as antioxidant. The functional assays were repeated with different yeast colonies to confirm the results.

Fatty acid analysis of yeast

FA from yeast total lipids were analysed by preparing methyl esters (FAME) as previously described (Hastings et al., 2001). FAME were identified and quantified using an Agilent 6850 Gas Chromatograph system coupled to a 5975 series MSD (Agilent Technologies, Santa Clara, CA, USA). The desaturation or elongation conversion efficiencies from exogenously added PUFA substrates were calculated by the proportion of substrate FA converted to desaturated or elongated products as $[\text{product area}/(\text{product area} + \text{substrate area})] \times 100$. For the *Elov15* assays, some of the initial elongation products were further elongated. In those cases, the accumulated conversion rates were also calculated by summing the individual conversion rate for each particular product and also those for longer products (Monroig et al., 2012b). When further confirmation of double bond positions was required, FA picolinyl esters were prepared from FAME according to the methodology described by Destailats and Angers (2002) and modified according to Li et al. (2010).

*Tissue distribution of *fads2* and *elovl5* transcripts*

In order to determine the mRNA distribution of the meagre *fads2* and *elovl5*, reverse transcription PCR (RT-PCR) analyses were performed on cDNA samples from liver, gill, stomach, pyloric caeca, spleen, testicle, kidney, heart, muscle, gallbladder, skin, brain and eye. PCR conditions for both target genes consisted of a denaturing step at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min (GoTaq[®] Green Master Mix, Promega). Additionally, the expression of the housekeeping gene *β-actin* was determined to check the cDNA integrity. Details of primers used for RT-PCR are given in Table 1.

Materials

Hexadecatrienoic acid (16:3n-3) was purchased from Larodan (Larodan Fine Chemicals AB, Malmö, Sweden), while the remaining PUFA substrates, except stearidonic acid (18:4n-3), were from Nu-Chek Prep, Inc (Elysian, MN, USA). Stearidonic acid and chemicals used to prepare the *S. cerevisiae* minimal medium-^{uracil} were from Sigma-Aldrich, except for the bacteriological agar obtained from Oxoid Ltd. (Hants, UK).

Results

Meagre fads2 and elovl5 sequences and phylogenetics

The ORF of the meagre *fads2* consisted of 1,338 bp encoding a putative protein of 445 aa. The deduced aa sequence showed identity scores ranging from 71.0 to 84.5 % with other Fads2 from several fish (*O. masou*, *T. thynnus*, *Dicentrarchus labrax*, *G. morhua*, *S. chuatsi*, *S. aurata*). Particularly high identity (98.9 %) was shown between the meagre Fads2 and that of *N. mitsukurii*, which, like meagre, is a member of the Sciaenidae family. Lower identity scores (60.8 - 64.2 %) were obtained when the *A. regius* Fads2 was compared to other Fads2-like sequences from mammals (*Homo sapiens* and *Mus musculus*), bird (*Gallus gallus*) and

amphibians (*Xenopus laevis* and *X. tropicalis*). Three histidine boxes (HXXXH, HXXHH and QXXHH), a putative cytochrome b5-like domain and a heme-binding motif (HPGG) were identified in the deduced aa protein sequence.

The coding sequence of the meagre *elovl5* was shown to be 885 bp in length, encoding a protein of 294 aa. Pairwise comparison of the translated aa sequence showed the meagre Elov15 shared 99.3% identity to the orthologues from the sciaenid fish *L. crocea* and *N. mitsukurii*, 95.6 - 92.5% identity to those of *D. labrax*, *L. calcarifer* and *S. aurata*, 83.1-76.2 % identity to *S. salar*, *S. canaliculatus* and *Clarias gariepinus*. Compared to Elov15-like proteins from other vertebrate classes, the meagre Elov15 aa sequence showed identity scores ranging from 69.6 (*H. sapiens*) to 72.5 % (*G. gallus*). The meagre Elov15 possesses a diagnostic histidine box (HXXHH), and lysine (K) and arginine (R) residues at the carboxyl terminus (KKXRX). The sequences of the newly cloned *fads2* and *elovl5* cDNA were deposited in GenBank with accession numbers KC261978 and KC261977, respectively.

The identity scores described above were reflected in the phylogenetic tree constructed on the basis of aa sequence comparisons of the meagre Fads2 (Fig. 1) and Elov15 (Fig. 2) with other homologous proteins from fish and other vertebrate species. Thus, the meagre Fads2 clustered with Fads2 from fish and more distantly with Fads2 from mammals, bird and amphibians. Additionally, Fads1-like proteins, a ‘front-end’ desaturase that is not present in teleost fish (Castro et al., 2012), grouped separately from the Fads2 cluster where meagre desaturase was included (Fig. 1). On the other hand, the meagre Elov15 grouped together with other teleostei orthologues, among which it formed a separate subgroup with Elov15 from *L. crocea* and *N. mitsukurii* (Fig. 2). Moreover, members of Elov12 and Elov14 families from fish and other vertebrates clustered more distantly from Elov15 proteins.

(FIGURE 1)

(FIGURE 2)

Functional characterisation in yeast

The meagre Fads2 and Elovl5 were functionally characterised by expressing their coding sequences in yeast *S. cerevisiae*. The FA profile of control yeast showed the main endogenous FA of *S. cerevisiae*, namely 16:0, 16:1 isomers (16:1n-9 and 16:1n-7), 18:0, 18:1n-9 and 18:1n-7 (Monroig et al., 2010a), plus one additional peak corresponding to the PUFA exogenously added as substrate (data not shown). This indicated that wild-type yeast do not express activities towards PUFA substrates, in agreement with earlier observations (Hastings et al., 2001; Agaba et al., 2004). Additional peaks were observed in the FA profiles of pYES2-*fads2* grown in the presence of 18:3n-3, 18:2n-6, 20:3n-3 and 20:2n-6, which were desaturated to 18:4n-3, 18:3n-6, 20:4n-3 and 20:3n-6, respectively (Fig. 3). These results suggest that the meagre Fads2 is a dual $\Delta 6/\Delta 8$ desaturase, with a relative $\Delta 6/\Delta 8$ activities towards n-3 substrates (18:3n-3 conversion vs. 20:3n-3 conversion) of 2.5. In contrast, the meagre Fads2 did not exhibit activity towards $\Delta 5$ - (20:4n-3 and 20:3n-6) or $\Delta 4$ - (22:5n-3 and 22:4n-6) desaturase FA substrates (Table 2). Although the conversion rates observed for the meagre desaturase were generally low compared to previous experiments conducted in our laboratories, the rates for the meagre Fads2 were confirmed in a second assay carried out with a different yeast colony (data not shown).

(TABLE 2)

(FIGURE 3)

Greater conversion rates were obtained in the functional characterisation assay run for the meagre Elovl5. Transgenic yeast expressing the coding sequence of the meagre *elovl5* showed activity towards most of the PUFA substrates assayed, with particularly high conversion rates for C18 and C20 substrates. Thus, C18 (18:3n-3, 18:2n-6, 18:4n-3 and 18:3n-6) and C20 (20:5n-3 and 20:4n-6) substrates were elongated to C20 (20:3n-3, 20:2n-6, 20:4n-3 and 20:3n-6) and C22 (22:5n-3 and 22:4n-6) products, respectively (Table 3; Fig. 4). Additionally, transgenic yeast expressing the *elovl5* ORF efficiently elongated 16:3n-3 up to 86 % of total substrate available (Table 3). For each pair of homologous substrates considered conversion

rates obtained from the yeast assays suggested that the meagre Elov15 elongated more efficiently n-3 than n-6 PUFA (Table 3). Thus, the substrates 18:3n-3, 18:4n-3 and 20:5n-3 were elongated at higher rates than the corresponding n-6 PUFA substrates 18:2n-6, 18:3n-6, 20:4n-6, respectively. C22 PUFA substrates including 22:5n-3 and 22:4n-6 were only marginally or not converted to longer products (Table 3). Other additional peaks including 20:1n-9 and 20:1n-7 (e.g., see peaks between retention times of 18:3n-3 and 20:3n-3 in panels A and B of Fig. 4) indicated some capability of the meagre Elov15 to elongate yeast endogenous monounsaturated FA, as previously described for the Atlantic salmon orthologues (Hastings et al., 2005; Morais et al., 2009).

(TABLE 3)

(FIGURE 4)

Tissue distribution of fads2 and elov15 transcripts

Tissue distribution of *fads2* and *elov15* mRNA in adult meagre was analysed by RT-PCR. The desaturase *fads2* transcripts were detected in all tissues analysed, with increased expression signal in brain compared to liver and digestive organs like pyloric caeca and stomach (Fig. 5). Similarly, the expression pattern of the *elov15* showed a widespread distribution in all tissues analysed, with apparent higher expression signals in brain, eye, liver and pyloric caeca (Fig. 5).

(FIGURE 5)

Discussion

The sequence analysis of the newly cloned Fads2- and Elov15-like cDNAs from *A. regius* confirmed they possess all the features for these enzymes. The Fads2 has three histidine boxes HXXXH, HXXHH and QXXHH common among ‘front-end’ desaturases, a putative cytochrome b₅-like domain, and the heme-binding motif, HPGG (Hashimoto et al., 2008). In addition, deduced aa sequence of the meagre Elov15 contained the diagnostic histidine box

(HXXHH) conserved in all members of the Elovl protein family, and lysine (K) and arginine (R) residues at the carboxyl terminus (KKXRX), regarded as putative ER retrieval signals (Jakobsson et al., 2006; Leonard et al., 2004). Functional characterisation by heterologous expression in *S. cerevisiae* confirmed the functional activities with the Fads2 desaturase possessing $\Delta 6$ and $\Delta 8$ activity and the Elovl5 having the ability to elongate C₁₆, C₁₈ and C₂₀ PUFA.

Compared to freshwater species, marine fish have been regarded as having low capability for LC-PUFA biosynthesis. The high availability of C₂₀₋₂₂ LC-PUFA in marine food webs was speculated to be a major evolutionary driving force that has resulted in the loss of specific enzymatic activities of the LC-PUFA pathway in marine fish species (Tocher, 2010). Put another way, there was no evolutionary pressure to maintain the LC-PUFA biosynthetic pathway in marine fish. However, this assumption had been based upon studies on marine species occupying high trophic levels in comparison to relatively lower trophic level of fish, with omnivorous and herbivorous feeding strategies, from freshwater environments (Tacon et al., 2010). The influence of ‘trophic level’ on the LC-PUFA biosynthetic capability of teleost fish species was demonstrated in the marine fish *Siganus canaliculatus*, a true herbivore that was shown to possess all the enzymatic activities required for the biosynthesis of C₂₀₋₂₂ LC-PUFA from C₁₈ PUFA precursors (Li et al., 2010; Monroig et al., 2012b). More recently, Morais et al. (2012) showed that ‘trophic ecology’ may also be a factor contributing to and/or driving the endogenous ability for LC-PUFA biosynthesis, with marine species like Senegalese sole (*Solea senegalensis*) showing unexpected ability for endogenous production of DHA from EPA which, nonetheless, was consistent with its EFA requirements. Therefore, the ability of different fish species to biosynthesise LC-PUFA appears to be determined by a complex range of factors.

The present study in meagre has provided further insight into the endogenous ability for LC-PUFA biosynthesis in marine fish. As well as being a new species for aquaculture,

meagre was also interesting to study as it displays anadromous behaviour, yet it is very different to Atlantic salmon and spends notably less time in the freshwater environment (river estuaries) (Monfort, 2010). Despite spending most of its life cycle in the sea, Atlantic salmon displays ‘freshwater’ characteristics as it has a proven LC-PUFA biosynthetic capability at both biochemical (Tocher et al., 2001; Zheng et al., 2005b) and molecular (Carmona-Antoñanzas et al., 2011; Hastings et al., 2005; Monroig et al., 2010a; Morais et al., 2009; Zheng et al., 2004, 2005a) levels. In contrast, the data for meagre suggest it has a classical marine pattern.

The evolution history of fatty acyl desaturases and elongases has attracted the interest of researchers (Hashimoto et al., 2006, 2008; Shanklin et al., 2009; Sperling et al., 2003). Front-end desaturases involved in the biosynthesis of LC-PUFA in vertebrates are of particular interest (Meesapyodsuk and Qiu, 2012) and, recently, Castro et al. (2012) established that all fatty acyl desaturases from teleost fish are orthologues of the mammalian *FADS2*, with *Fads1*-like desaturases only present in cartilaginous fish. Our results confirm that the meagre desaturase cDNA isolated in the present study encoded a *Fads2* desaturase. While the *Fads2* appears to be the sole fatty acyl desaturase existing in teleost fish, the functionalities of each specific *Fads2* protein present in different fish species has been shown to be more diverse than in other vertebrates. In mammals, *Fads1* desaturases have $\Delta 5$ activity, whereas *Fads2* enzymes are predominantly show $\Delta 6$ specificity (Guillou et al., 2010). In the majority of teleostei *Fads2* are also typically $\Delta 6$ desaturases (González-Rovira et al., 2009; Mohd-Yusof et al., 2010; Monroig et al., 2010; Morais et al., 2011; Santigosa et al., 2011; Tocher et al., 2006; Zheng et al., 2004, 2009) but, in addition, *Fads2*-like cDNAs encoding desaturases with $\Delta 5$ (Hastings et al., 2005), $\Delta 4$ (Li et al., 2010; Morais et al., 2012) and bifunctional $\Delta 6/\Delta 5$ (Hastings et al., 2001; Li et al., 2010) activities have also been identified. The functional assays for the meagre *Fads2* revealed that the enzyme has $\Delta 6$ -desaturase activity, with no $\Delta 5$ or $\Delta 4$ activity. Compared to most other teleost desaturases functionally characterised in our

laboratories, the conversion rates obtained for the meagre Fads2 were unexpectedly low, similar to those of two fatty acyl desaturases from Atlantic salmon, namely the $\Delta 5$ Fad (Hastings et al., 2005) and the $\Delta 6$ Fad_c (Monroig et al., 2010a). However, the data from heterologous expression systems like yeast, upon which substrate specificity of desaturases and elongases is inferred, should primarily be regarded as informative of the potential qualitative roles of the genes *in vivo*. Competence with other enzymes and different substrate availability *in vivo* might substantially change the quantitative conversions compared to those observed *in vitro*. However, due to the lack of endogenous PUFA converting enzymes, yeast have been the expression system of choice for functional characterisation of enzymes involved in the biosynthesis of LC-PUFA from a large variety of organisms (Gregory et al., 2013; Huang et al., 1999; Jiang et al., 2011; Leonard et al., 2002; Qiu et al., 2001; Sayanova et al., 2006, 2011; Watts and Browse, 1999).

In addition to $\Delta 6$ -desaturation ability, the meagre Fads2 was also able to desaturate the C₂₀ PUFA substrates 20:3n-3 and 20:2n-6 to 20:4n-3 and 20:3n-6, respectively, thus indicating $\Delta 8$ -desaturase activity, a common trait among fish Fads2 (Monroig et al., 2011a). The $\Delta 6/\Delta 8$ bifunctionality of the Fads2 enables the biosynthesis of LC-PUFA from ALA and LOA in meagre to proceed via two alternative routes: the ‘ $\Delta 6$ pathway’, with ALA and LOA initially desaturated at the $\Delta 6$ position to 18:4n-3 and 18:3n-6, respectively; and the ‘ $\Delta 8$ pathway’, by which an initial elongation of ALA and LOA produces 20:3n-3 and 20:2n-6, respectively, which can be subsequently desaturated at the $\Delta 8$ position to 20:4n-3 and 20:3n-6. Irrespective of which of these pathways is taken, the biosynthesis of EPA and ARA still requires a further desaturation at the $\Delta 5$ position of 20:4n-3 and 20:3n-6, respectively, a desaturation activity that was not identified in the meagre Fads2. Indeed the low efficiency of marine fish for biosynthesis of LC-PUFA has been often hypothesised to be due to the lack of $\Delta 5$ -desaturase (Tocher, 2003, 2010), rather than limited $\Delta 6$ -desaturase as occurs in mammals (Aki et al., 1999; Cho et al., 1999). Notably, the $\Delta 6/\Delta 8$ ratio of the meagre Fads2, i.e. the ratio of the

410 conversion rates towards 18:3n-3 and 20:3n-3, was 2.5, falling within the range of $\Delta 6/\Delta 8$
411 ratios of marine fish desaturases (1.8-4.2) and much lower than those of desaturases from
412 diadromous/freshwater species (12.0-91.2) (Monroig et al., 2011a). Further evidence
413 supporting the assertion that meagre has a 'marine type' EFA requirement pattern was
414 provided by the tissue distribution of the desaturase. Thus, brain appeared as the major
415 biosynthetic site over liver and intestinal organs (pyloric caeca and stomach), a pattern
416 similarly observed in other marine fish like Asia sea bass, Atlantic cod and cobia (Mohd-
417 Yusof et al., 2010; Tocher et al., 2006; Zheng et al., 2009), and different to
418 freshwater/salmonid species in which liver and intestine exhibit highest expression signals of
419 key desaturase and elongase genes (Zheng et al., 2005a; Morais et al., 2009).

420 Unlike desaturation, all the elongation activities required for the production of EPA and
421 ARA from C₁₈ PUFA are confirmed to exist in meagre. Thus, the meagre Elov15 efficiently
422 elongated a range of C₁₈ PUFA. Firstly, it was able to elongate 18:4n-3 and 18:3n-6 to 20:4n-
423 3 and 20:3n-6, respectively, as required for the 'classical' $\Delta 6$ -pathway. Secondly, the Elov15
424 was able to elongate ALA and LOA to 20:3n-3 and 20:2n-6, respectively, the latter being the
425 C₂₀ substrates of the Fads2 in the ' $\Delta 8$ pathway'. While the first alternative has been
426 demonstrated for the majority of Elov15-like proteins of fish so far studied (Agaba et al.,
427 2004, 2005; Zheng et al., 2009; Morais et al., 2009, 2011; Mohd-Yusof et al., 2010; Monroig
428 et al., 2012), only a few studies have assayed the ability of teleost fish Elov15 to operate
429 towards 18:3n-3 and 18:2n-6 including those on Southern bluefin tuna (Gregory et al., 2010)
430 and Northern pike (Carmona-Antoñanzas et al., 2013). These results support the 'plasticity' of
431 fish elongases, which generally exhibit wider PUFA specificities than their homologs from
432 higher vertebrates (Morais et al., 2009). Like other fish Elov15, the meagre Elov15 showed
433 high elongation efficiencies towards C₁₈ and C₂₀ substrates, but also a low activity towards
434 C₂₂ PUFA (Agaba et al., 2004, 2005; Zheng et al., 2009; Morais et al., 2009, 2011; Gregory et
435 al., 2010; Mohd-Yusof et al., 2010; Monroig et al., 2012). In contrast, mammalian ELOVL5

also showed high activity towards C₁₈ and C₂₀ PUFA, but were unable to elongate C₂₂ PUFA substrates (Leonard et al. 2000; Inagaki et al. 2002). Some Elovl5 of other fish species have relatively high capacity for elongation of C₂₂ PUFA with the rabbitfish *S. canaliculatus* Elovl5 converting up to 10.6 % of 22:5n-3 to 24:5n-3 (Monroig et al., 2012b). Moreover, another member of the vertebrate elongase family, Elovl4, in particular isoform b (Elovl4b), exhibited the ability to elongate PUFA with chain-lengths ranging from C₂₀ and C₃₄ in several teleost species (Monroig et al., 2010b, 2011b, 2012b; Carmona-Antoñanzas et al., 2011). Human ELOVL4 showed some limited capacity for elongation of C₂₆ PUFA (Agbaga et al., 2008).

While the structure-function relationships underpinning the ability of fish Elovl enzymes to operate towards a wide range of fatty acyl substrates remain unknown, here we report for the first time that a teleost Elovl5 was able to efficiently elongate C₁₆ PUFA. Clearly, the results demonstrated that the meagre Elovl5 elongated 16:3n-3 with the highest conversion rate (86 % of total 16:3n-3 incorporated into yeast) of all PUFA substrates assayed. The ability of the meagre Elovl5 to elongate 16:3n-3 may reveal an inherent ability of fish to efficiently utilise C₁₆ PUFA to produce C₁₈ and C₂₀ PUFA that can be subsequently incorporated into the ‘classical’ biosynthetic pathways to EPA and DHA. The fatty acid 16:3n-3 is naturally produced in many plants and is generally found in the marine environment (Ackman, 1980). Thus, seaweeds of the genus *Caulerpa* (Khotimchenko, 1995) and *Codium* (Goecke et al., 2010), seagrasses of the Zosterioideae family (Khotimchenko, 1993) and leaves of the so-called “16:3 plants” like *Arabidopsis*, rapeseed and spinach (Browse and Somerville, 1991; Wallis and Browse, 2002), among others, may represent interesting alternative sources of dietary FA for aquafeeds. Like the vast majority of fish Elovl5, the meagre Elovl5 was more efficient towards n-3 than n-6 PUFA substrates, when metabolically equivalent pairs were compared.

In conclusion, the present study has provided evidence that meagre expresses at least one fatty acyl desaturase and one elongase involved in the endogenous production of LC-PUFA. The desaturase was a 'front-end' desaturase with homology to Fads2 and confirmed to have dual $\Delta 6/\Delta 8$ activities. The fatty acyl elongase was demonstrated to be an Elovl5 enzyme commonly found in teleost fish. The data confirmed that the meagre Elovl5 had C₁₈ and C₂₀ PUFA as preferred substrates, but also low activity towards C₂₂ PUFA. These characteristics were similar to other marine teleost fish. However, we further demonstrated, for the first time, that a marine fish Elovl5 elongated 16:3n-3 to 18:3n-3, suggesting that alternative sources containing 16:3n-3 may be utilised in aquafeeds.

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Legends to Figures

Fig. 1. Phylogenetic tree comparing the deduced amino acid (aa) sequence of the newly cloned *Argyrosomus regius* fatty acyl desaturase (Fad) 2-like with Fads1- and Fads2-like desaturases from a variety of vertebrates. The tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with MEGA4. The horizontal branch length is proportional to aa substitution per site. The numbers represent the frequencies with which the tree topology presented was replicated after 10,000 bootstrap iterations. All accession numbers are from GenBank database.

Fig. 2. Phylogenetic tree comparing the deduced amino acid (aa) sequence of the newly cloned *Argyrosomus regius* elongase of very long-chain fatty acids (Elovl) with elongases Elovl2, Elovl4 and Elovl5 from a variety of vertebrates. The tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with MEGA4. The horizontal branch length is proportional to aa substitution per site. The numbers represent the frequencies with which the tree topology presented was replicated after 10,000 bootstrap iterations. All accession numbers are from GenBank database.

Fig. 3. Functional characterisation of the of the newly cloned *Argyrosomus regius* Fads2 in yeast (*Saccharomyces cerevisiae*). The fatty acid profiles of yeast transformed with pYES2 containing the coding sequence of the meagre Fads2 as an insert, were determined after the yeast was grown in the presence of one of the exogenously added substrates 18:3n-3 (A) and 20:3n-3 (B). Peaks 1-5 in panels A and B are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1 isomers (2), 18:0 (3), 18:1n-9 (4) and 18:1n-7 (5). Additionally peaks derived from exogenously added substrates (“*”) and desaturated products are indicated accordingly in panels A-B. Vertical axis, FID response; horizontal axis, retention time.

Fig. 4. Functional characterisation of the of the newly cloned *Argyrosomus regius* elongase of very long-chain fatty acids 5-like (Elovl5) in yeast (*Saccharomyces cerevisiae*). The fatty acid profiles of yeast transformed with pYES2 containing the coding sequence of Elovl5 as an insert, were determined after the yeast was grown in the presence of one of the exogenously added substrates 16:3n-3 (A), 18:3n-3 (B), 18:4n-3 (C) and 20:5n-3 (D). Peaks 1-5 in panels A-D are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1 isomers (2), 18:0 (3), 18:1n-9 (4) and 18:1n-7 (5). Additionally peaks derived from exogenously added substrates (“*”) and elongated products are indicated accordingly in panels A-D. Vertical axis, FID response; horizontal axis, retention time.

Fig. 5. RT-PCR analyses showing the tissue distribution of *Argyrosomus regius* fatty acyl desaturase *fads2* and elongase of very long-chain fatty acids 5 (*elovl5*) transcripts. Expression of the housekeeping gene *β-actin* is also shown. NTC, no template control.

Tables

Table 1. Sequences of the primer pairs used and accession numbers of the sequences used as references for primer design in the cloning of the meagre *fads2* and *elovl5* ORF and for RT-PCR analysis of gene expression in tissues.

Aim	Transcript	Primer	Primer sequence	Accession No ¹ .
3' RACE PCR	<i>fads2</i>	ARDF1	5'-TACATTTCGCTTCCTGTGCTG-3'	KC261978
		ARDF2	5'-GGAGAGTCACTGGTTTGTGTG-3'	
	<i>elovl5</i>	ARE5F1	5'-CATCACACAGTTACAGCTGGTC-3'	KC261977
		ARE5F2	5'-CCATGTCCCAGACTATGTGC-3'	
5' end PCR	<i>fads2</i>	NMDesF	5'-ATGGGAGGTGGAGGCCAG-3'	GQ996729
		ARDR	5'-AACAAAGGCTGAGCTCGAAA-3'	
	<i>elovl5</i>	LCE5F	5'-ATGGAGACCTTCAATCATAAAC-3'	JQ320377
		ARE5R	5'-AGTGTGAGGCCAGATTGT-3'	
ORF cloning	<i>fads2</i>	ARDVF	5'-CCC <u>GAATTC</u> AAAAATGGGAGGTGGAGGCCA-3'	KC261978
		ARDVR	5'-CCG <u>CTCGAG</u> GGTCATTTGTGGAGATATGCATC-3'	
		ARDU3R	5'-TGGTGCTAACTTTGTGCCCT-3'	
	<i>elovl5</i>	ARE5VF	5'-CCC <u>GAATTC</u> AAAAATGGAGACCTTCAATCAT-3'	KC261977
		ARE5VR	5'-CCG <u>CTCGAG</u> GTCAATCCACCCTCAGTT-3'	
		ARE5U3R	5'-GAATTGTGTGCACGGTTTCT-3'	
RT-PCR tissue expression	<i>fads2</i>	ARDF3	5'-TGA CTGGGTGACAATGCAGT-3'	KC261978
		ARDU3R	5'-TGGTGCTAACTTTGTGCCCT-3'	
		ARE5F3	5'-CATCACACAGTTACAGCTGGTC-3'	
	<i>elovl5</i>	ARE5U3R	5'-GAATTGTGTGCACGGTTTCT-3'	KC261977
	β -actin	UNIActF	5'-CCATCGAGCACGGTATTGT-3'	GU584189
		UNIActR	5'-CAGCTTCTCCTTGATGTCACG-3'	

¹ GenBank (<http://www.ncbi.nlm.nih.gov/>)

751 Table 2. Functional characterisation of the meagre Fads2 in *Saccharomyces cerevisiae*.
 752 Results are expressed as a percentage of total fatty acid (FA) substrate converted to
 753 desaturated product.

FA substrate	Product	% Conversion	Activity
18:3n-3	18:4n-3	5	Δ6
18:2n-6	18:3n-6	3	Δ6
20:3n-3	20:4n-3	2	Δ8
20:2n-6	20:3n-6	1	Δ8
20:4n-3	20:5n-3	0	Δ5
20:3n-6	20:4n-6	0	Δ5
22:5n-3	22:6n-3	0	Δ4
22:4n-6	22:5n-6	0	Δ4

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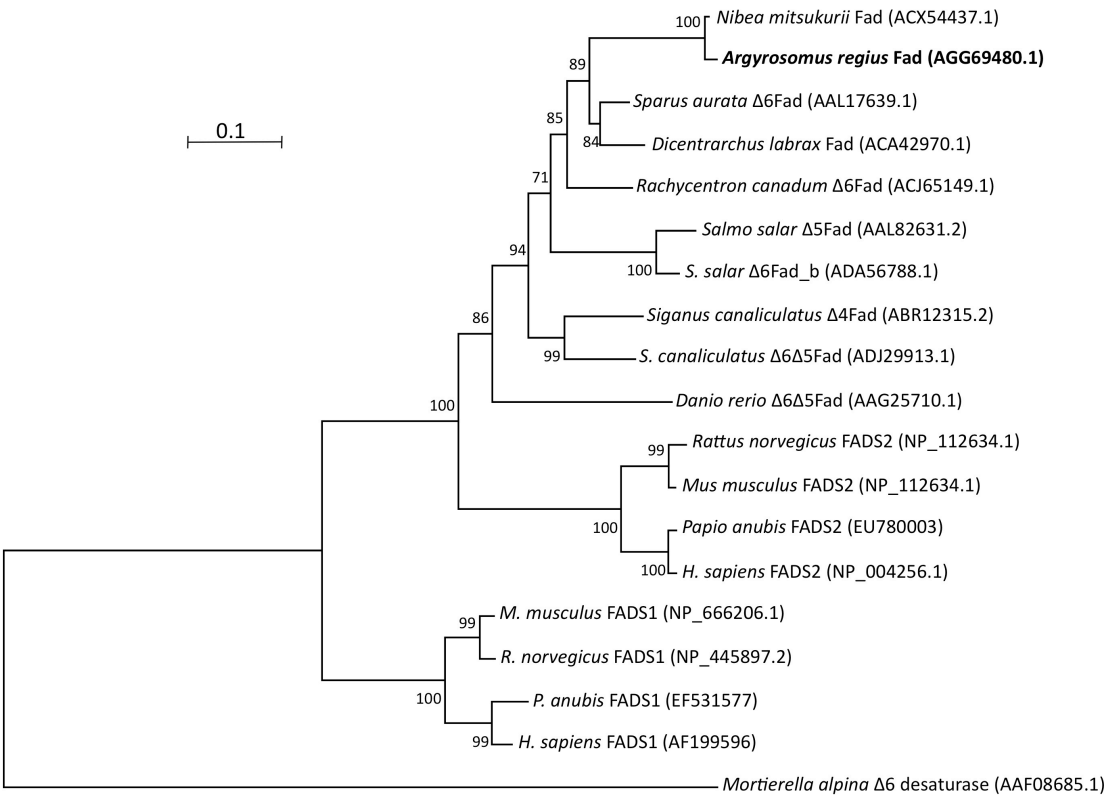
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756 Table 3. Functional characterisation of meagre Elovl5 in *Saccharomyces cerevisiae*.
757 Individual conversion rates were calculated according to the formula [individual product
758 area/(all products areas + substrate area)] x 100. Accumulated conversions were computed by
759 summing the individual conversion rate for each particular product and also those for longer
760 products.

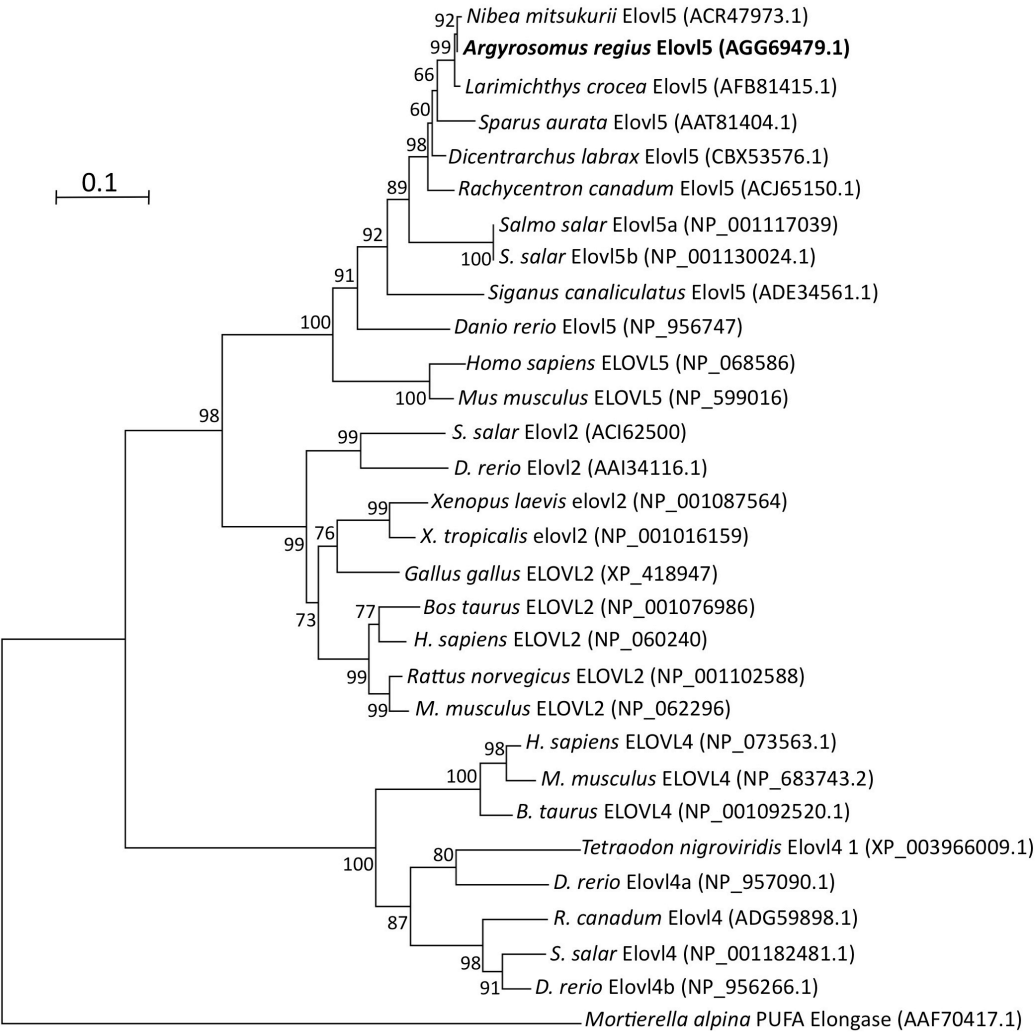
Fatty acid substrate	Product	% Individual conversion	% Accumulated conversion
16:3n-3	18:3n-3	62	86
	20:3n-3	24	24
18:3n-3	20:3n-3	33	34
	22:3n-3	1	1
18:2n-6	20:2n-6	17	17
18:4n-3	20:4n-3	66	77
	22:4n-3	11	11
18:3n-6	20:3n-6	61	68
	22:3n-6	7	7
	24:3n-6		
20:5n-3	22:5n-3	65	68
	24:5n-3	3	3
20:4n-6	22:4n-6	59	59
22:5n-3	24:5n-3	2	2
22:4n-6	24:4n-6	0	0

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763 Fig. 1.
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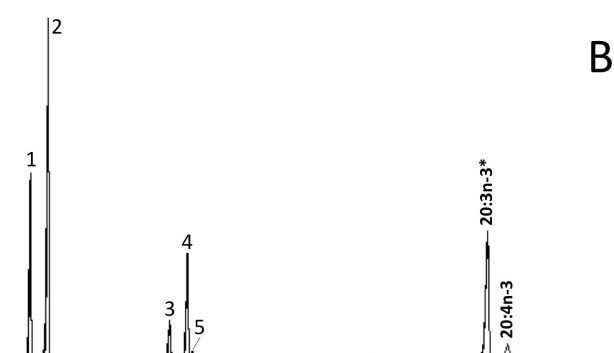
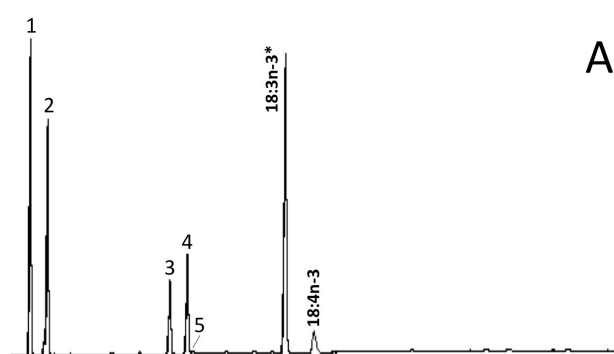


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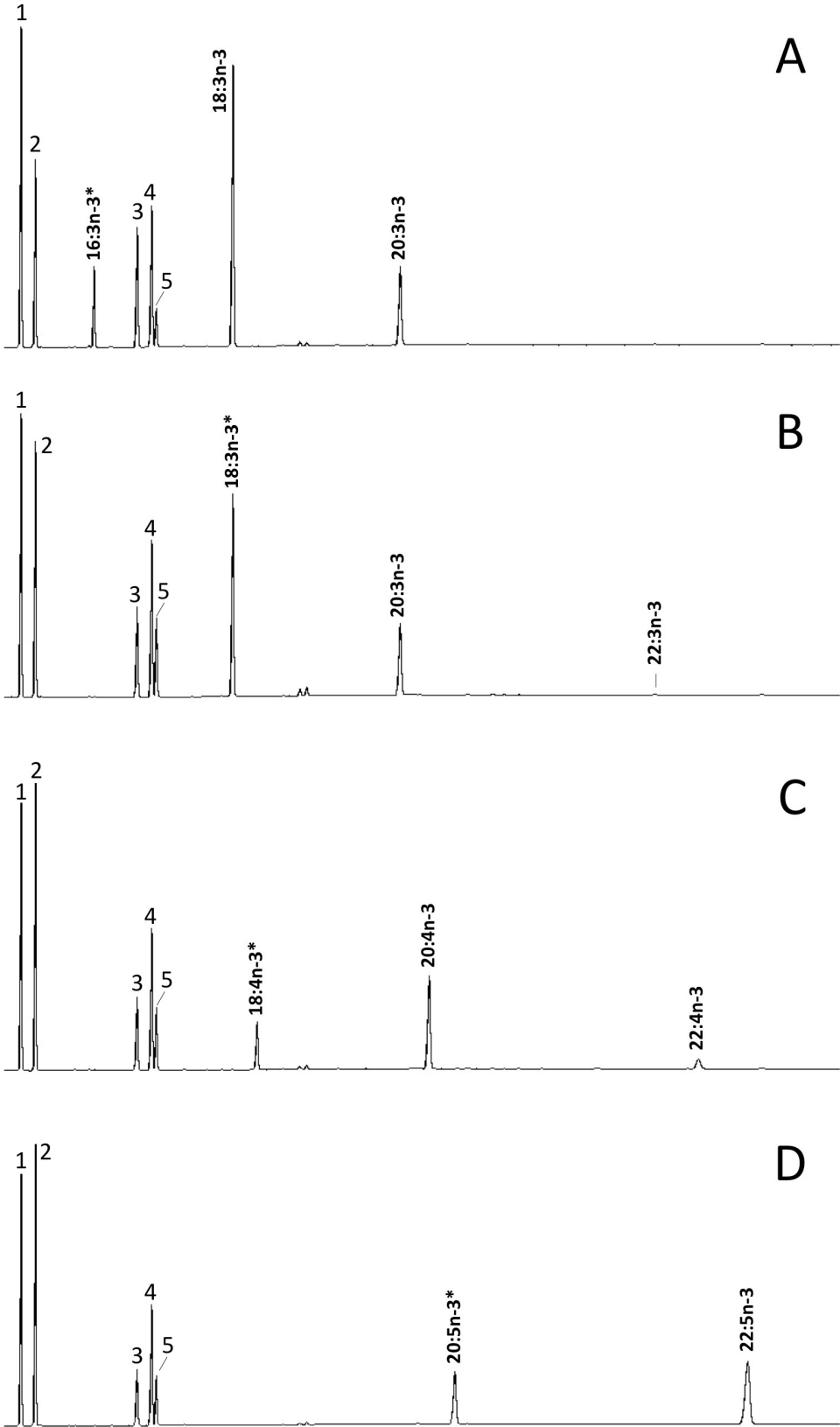
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771 Fig. 3.

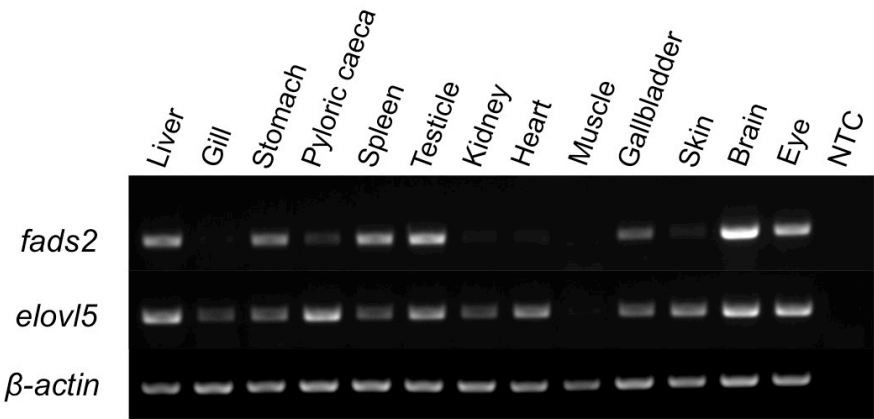


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777 Fig. 5.



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